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THE EFFECTS OF SUBCUTANEOUS SALINE INJECTIONS ON GROWTH AND KERATINIZATION OF MOUSE TAIL EPIDERMIS*

R. I. C. SPEARMAN, B.Sc., PH.D. AND M. GARRETTS, M.B., B.S., M.R.C.P.

Regional differences in keratinization are partly determined by the underlying dermis as it has been shown to induce keratinization in embryonic chick epithelium, and there is probably a similar mechanism in adult skin (1, 2, 3, 4).

Keratinization is also influenced by changes in epidermal cell proliferation. This is evidenced by the fact that increased mitosis in mammalian epidermis generally results in the loss of a granular layer and a change from normal keratinization to a scaly parakeratotic horny layer (4).

Keratinization can also be altered experimentally by physical agents. Thus, rubbing, inserting a needle, or scratching, all stimulate epidermal mitosis (5, 6), and tend to cause parakeratosis (7, 8). In contrast, the induction of a granular layer in the mouse tail scale region by vitamin A was shown to be independent of the change in epidermal proliferation (4).

Menkin (9) reported a growth substance produced by dermal inflammatory cells which affects the epidermis. The epidermis also becomes thickened and hyperkeratotic as a result of prolonged edema, but the reason for this has not yet been explained (10).

In the present investigation, the effects of subepidermal injections of isotonic saline on growth and keratinization of mouse tail epidermis were examined. Injections were given daily for one month, and a temporary local edema was produced on each occasion. The animals were killed and the tail skin examined for histological changes and especially for any alteration in keratinization.

A second paper deals with the rate of removal of the injected saline from the skin (11).

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* From the Department of Dermatology, University College Hospital Medical School, London and the Manchester Royal Infirmary, Manchester, England.

MATERIALS AND METHODS

The normal mouse tail epidermis: Epidermal keratinization in the mouse tail skin differs from that in other regions in that square horny scales alternate with hair follicles (Fig. 1). The scales are arranged in rings around the tail. Adjacent scale rings are, however, out of phase so that interscale regions of one ring are opposite the mid-scale regions of the adjacent rings (12).

In the house mouse, a granular layer is formed in the epidermis around the hair follicles and in the epidermis lateral to each scale, but a granular layer does not develop in the scale regions. Marked differences can be detected in the chemical constituents and in keratin fluorescence between horny layers formed by way of a granular layer and those which are formed without such a layer. Thus the normal mouse tail scales fluoresce blue with congo red and contain bound phospholipids and sulfhydryl groups, whereas the horny layer around the hair follicle which is formed from a granular layer fluoresces red with congo red and has relatively few phospholipids and sulfhydryl groups (4, 8).

Injection procedure: Sterile 0.9 per cent saline at pH 6 and at 4° C. was injected daily for 30 days into the dermis of the tails of male albino mice weighing about 30 grams. The needle was inserted at the base of the tail and directed parallel to the skin surface towards the tip for a distance of about two centimeters. This was to avoid, as far as possible, epidermal trauma over the site where the edema was produced. Sufficient saline was slowly injected to produce blanching of a 2 cm length of skin. By this means it was possible to produce a transient edema with about 0.2 ml saline without rupturing the epidermis overlying the saline bleb. The fluid spread easily in the dermis so that the tail was encircled by the bleb. Deeper injections into the skin met considerable resistance, and it was impossible to blanch the overlying dermis.

Control mice received 0.2 ml saline into the back skin, but this was insufficient to produce a bleb. In the same animals a syringe needle was also inserted daily into the tail to produce trauma; no fluid was injected. All injections were performed under light ether anesthesia.

Histological technic: The animals were killed 24 hours after the last injection. The tail skin was removed and the first two cm from the base of the tail were rejected. The flattened skin was then fixed in 70 per cent ethanol. Paraffin sagittal sections of tail skin were prepared from the same region in which the saline blebs had been pro-

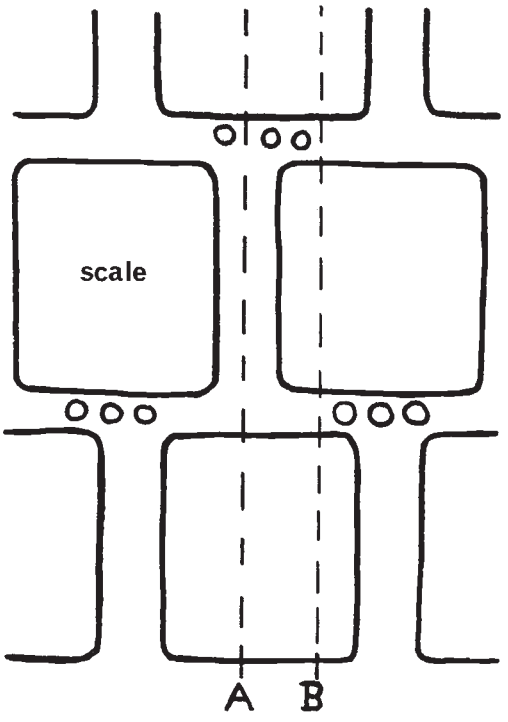

FIG. 1. The house mouse tail scale pattern showing groups of hairs behind each scale. Sagittal sections through A were rejected for histology as they passed alternately through scales without a granular layer and lateral interscale regions with a granular layer. Sections through B were selected for study as they included only scale and follicular regions.

duced. Sections were stained in hematoxylin and eosin and by the Congo red method for fluorescence microscopy.

Histochemical methods: Bound phospholipids were examined by the acid hematin method (8, 13). This was a modification of Baker's original technic (14).

Bound sulfhydryl groups of cysteine were examined by the D.D.D. method of Barmett and Seligman (15) and alkaline phosphatase was shown by Gomori's silver method (16).

Epidermal thickness: This was measured in the mid-scale regions from the basal layer to the underside of the horny layer. The mean epidermal thickness was then calculated from ten neighboring scale measurements and the standard deviation determined.

Mitotic counts: Fifteen mice were treated for 30 days with daily subcutaneous saline injections into the tail dermis, and a further five control mice were given saline into the back skin and the needle merely inserted into the tail. All the mice were killed at (3.0 pm) five hours after an injection of 0.1 mg colchicine in water for 100 g body weight (17).

The arrested metaphase nuclei were counted in eight 0.5 mm lengths of epidermis and the mean was calculated. The sample mean and standard deviation were then determined.

RESULTS

Effects of Saline Injections on the Tail Skin

After one week, the epidermis over the injected area appeared rough and horny. Compression of the superficial vessels, shown by blanching, lasted for up to 30 minutes following each injection.

(a) *Histological changes in the skin.*—Sagittal sections of tail skin from 33 mice after the saline treatment showed a thickened epidermis and a thickened granular layer in the scale regions as well as around the follicles. The granular layer was up to four cells thick, as compared with that around normal mouse tail follicles which is only one cell in thickness. The tail scale and follicular horny layers were thicker than normal, but parakeratosis did not occur. The only change observed in the dermis of altered tails was a deeper basophilic staining of the superficial dermal cells.

(b) *Tail scale epidermal thickness.*—

Saline-treated tails
53.5 μ ± S.D. 11.6 (N = 33)
Controls
28.2 μ ± S.D. 2.2 (N = 9)

The increase in epidermal thickness after saline injection was, therefore, highly significant.

(c) *Fluorescence microscopy (Congo red method).*—The appearance was quite different from the normal mouse tail. In the saline-treated mice the horny layer in both the scale and follicular regions showed alternate layers of blue and red fluorescing keratin. These lamellae were sharply defined, and no intermediate keratin color occurred.

Normal epidermal cell cytoplasm fluoresces red by this technic, and the nuclei are yellow. In the saline-treated tails occasional epidermal cells well below the granular layer appeared to have keratinized prematurely and had a blue cytoplasm. Comparison with hematoxylin and eosin stained sections showed similar eosinophilic cells.

Fluorescence with thioflavine T, before and after RNase and DNase digestion, suggested that both DNA and RNA were increased in the

epidermal cells as compared with normal control animals. A similar change was detected in the cells of the superficial dermis, which accounts for their basophilia.

(d) *Bound phospholipids.*—In the saline-treated tails there were similar alternate layers of keratin showing the presence and absence of bound phospholipid in the follicular and scale regions. The distribution of bound phospholipids, therefore, closely followed the distribution of the blue fluorescing keratin as seen with the Congo red method. The few individual cells which keratinized prematurely also reacted for bound phospholipids. This differed from normal and control mice in which bound phospholipids were largely confined to the tail scales.

(e) *Bound sulfhydryl groups.*—There were strongly positive patches in both the follicular and scale keratins, in contrast to normal tail skin in which the reaction is strongest in the scale keratin.

(f) *Alkaline phosphatase.*—Vitamin A applied locally to the skin produces marked alkaline phosphatase activity in the thickened epidermis (4). The thickened saline-treated tail skin was, therefore, examined for the presence of this enzyme by the same silver technic (15). However, although control skin treated with vitamin A showed a strong reaction, none was detected in saline-treated skin even though there was considerable hyperplasia.

Control Animals

Histological examination of the back skin of the controls failed to show any change. Neither was any histological change observed in the tail as a result of the insertion of a needle into the epidermis and dermis. The distribution of keratin bound phospholipids and sulfhydryl groups were the same as in untreated animals.

Epidermal Mitotic Activity

The saline treatment caused a significant increase in epidermal mitosis over the injection site. The number of arrested epidermal metaphase nuclei were as follows:

Saline-treated tails
4.5 ± S.D. 0.8 (N = 15)
Control tails
2.4 ± S.D. 0.5 (N = 5)

RESPONSE AFTER TRIAMCINOLONE AND SALINE INJECTIONS INTO THE TAIL

In a further experiment saline was injected daily into the tails of 11 mice for 30 days and in addition a suspension of triamcinolone in water (0.25 mg per 30 g body weight) was injected daily subcutaneously into the back.

Triamcinolone is a prednisolone derivative having a fluorine atom in its molecule. This corticosteroid depresses mitosis and reduces the thickness of normal mouse tail epidermis and stimulation by vitamin A (4). It was, therefore, desirable to find out whether it would also depress the epidermal hyperplasia due to saline injections.

(a) *Histology.*—The effect of the additional treatment with triamcinolone was inconclusive. In seven mice the follicular and tail scale regions showed the same stratified keratin effect as in the series with saline alone. In two animals the tail scale and follicular regions were mainly red, and in two mice the epidermis was not thickened.

(b) *Tail scale epidermal thickness.*—

Saline alone
53.5 μ ± S.D. 11.6 (N = 33)
Saline and systemic triamcinolone
47.5 μ ± S.D. 13.7 (N = 11)

Systemically administered triamcinolone failed to produce a significant reduction in the saline induced epidermal hyperplasia.

DISCUSSION

Because even slight mechanical stimulation is known to increase epidermal mitosis, it is probable that the hydrostatic tension on the epidermis following saline injections accounts for the increased cell division. The increase in epidermal thickness appears to be partly due to increased proliferation, and partly to the hypertrophy of the epidermal cells. The epidermal thickness was not significantly reduced by even large doses of systemic triamcinolone. This indicates the powerful nature of the stimulus, as thickening of the epidermis due to vitamin A can be diminished by giving the same dose of steroid (4).

Usually a granular layer is not formed in an epidermis which is rapidly proliferating, and a parakeratotic horny layer is produced (19, 8). However, in the saline experiments a prominent

granular layer was formed in the tail scale epidermis where a granular layer is not normally found. The development of a granular layer in this abnormal situation is probably not merely due to the general increase in epidermal thickness.

Oleic acid causes thickening of the epidermis but fails to induce a granular layer in the scale regions (4) and, therefore, some additional effect of the saline, other than a stimulus to mitosis, must be responsible for this change.

The fluorescence of normal mouse tail skin with the congo red and thioflavine T technic has already been reported (8). The scale keratin is blue and the follicular keratin is red. In the saline-treated tails the normal keratin fluorescence was profoundly altered, so that two distinct types of horny layer developed in alternate layers. This periodic alternation which occurred in both the follicular and scale keratins is most likely associated with the periodicity of the daily injection stimulus.

It is reasonable to suppose that the red fluorescing keratin with a low phospholipid content is determined by the development of a granular layer in the scale regions. The blue fluorescing keratin, rich in phospholipids, is less readily explained, but it is possibly produced at times of peak epidermal proliferation immediately following an injection. Parakeratotic keratin shows a similar fluorescence but has stainable nuclei. These two effects, one inducing a granular layer, and the other stimulating mitosis, probably account for the stratified appearance of the tail horny layer.

A further effect of the saline was seen in the occasional premature keratinization of individual epidermal cells deep in the epidermis before they reached the granular layer. Little is yet known of the factors which determine the stage at which an epidermal cell keratinizes. Individual cell keratinization occurs in pathological conditions such as squamous cell carcinomas, and this is probably not directly related to an increased rate of proliferation.

Locally applied vitamin A also caused epidermal hyperplasia and induced a granular layer in the tail scale epidermis, but a stratified keratin layer was not produced (4). The horny layer was not so thickened as after the saline injections and individual cell keratinization did not occur.

Local blanching after injection of saline never lasted longer than thirty minutes, and this would suggest that the edema was extremely transient. (In another paper the rate of removal is more critically examined by using isotope labelled saline.)

Dermal Effects

Apart from the effect of hydrostatic pressure on mitosis, saline may indirectly affect the epidermis by a dermal action. The induction of a granular layer in the tail scale regions may possibly be due to upset of some normal dermal induction mechanism.

The only obvious direct effect the saline could have on the dermis is the alteration of its collagen gel structure. The hydrogen ion effect is unlikely to be important as the present experiments were carried out at pH 6 which does not alter the protein gel (20). A possible cause must, therefore, be the ions themselves. Of these, chloride ions are more likely to affect the collagen as they belong to the lyotropic series and cause swelling and solution of gels (21, 22). It is known that some of the collagen can be extracted from connective tissue by cold salt solutions (23). In our experiments the saline was kept at 4° C, and this may have helped this process. It is, however, difficult to see how a mere physical variation in the dermal collagen could be directly responsible for effects on the epidermis.

Changes in the collagen gel structure would affect the rate of diffusion through the dermis of any large molecular growth substances. It would also alter the environment of the dermal cells which may influence the epidermis. Normal saline is only physiological in that it is isotonic, as the ratio of Cl^- to Na^+ ions is different in saline to tissue fluids (24). This change in ionic concentration may affect the dermal cells.

Diffusion upwards of the sodium and chloride ions into the superficial dermis is probably fairly rapid. Thus Manegold and Kalauch (25) showed that diffusion of sodium and chloride ions through negatively charged gels with a relatively large pore size is as rapid as through water.

The epidermal changes which occur after saline injections are unlikely to be caused by the growth substance demonstrated by Menkin (9). This substance is produced in severe inflammatory states, and there was no sign of an

inflammatory reaction in the saline treated mouse tail skins.

Keratin changes as a result of prolonged edema

In view of the changes produced experimentally by saline injection it is possible that certain of the keratin changes which occur in prolonged edemic states, such as elephantiasis, may have a similar basis. However, little experimental work has been published on the subject.

The suggestion of Sämberger (26) that chronic hypernutrition as a result of building up of nutrients in the tissue fluid might cause epidermal hyperplasia seems untenable. In fact, nutrients are probably diminished in quantity owing to the poor circulation in the edemic area.

Importance of findings to experimental work on animals

The finding that isotonic saline injected into the mouse tail skin produced profound changes in the epidermis is important in assessing experimental findings with injected substances in general. It should be realized that when raised blebs of saline under tension are formed in the dermis that the possibility exists of producing similar results as in these experiments.

SUMMARY

1. The normal mouse tail epidermis develops a granular layer around the hair follicles and in the zone lateral to each scale. The tail scale regions do not form a granular layer. Epidermal keratinization is also different around the follicles to that in the scales. The perifollicular horny layer fluoresces red with Congo red and contains little phospholipid. The tail scales contain appreciable bound phospholipid and fluoresce blue with congo red.

2. The daily injection into the deep dermis of the tail of a small quantity of sterile isotonic saline without added preservative resulted after one month in a local alteration of epidermal growth and keratinization. Epidermal mitosis was increased, the epidermis was markedly thickened, and a prominent granular layer was formed in the tail scale region as well as around the follicles. The keratin fluorescence (and bound phospholipid distribution) was altered so that red and blue fluorescing keratins appeared in alternate layers in both the follicular and scale regions.

3. The increased epidermal mitosis is probably explained by the pressure stimulus on the epidermal basal cells. Trauma to the epidermis through the injection of too much saline was avoided. The syringe needle was inserted horizontally into the skin so that the site of insertion was away from the site of study.

4. The induction of a granular layer in the abnormal site of the tail scales and the keratinization change are not readily explained by the increase in epidermal mitotic rate.

5. Other possible effects of the saline on the dermis besides pressure which might indirectly affect the epidermis are discussed.

6. It is suggested that the keratin changes reported are partly due to the increased epidermal proliferation and partly to the induction of a new granular layer. Stratification of the keratins in the horny layer is probably related to the periodicity of the injection stimulus.

7. The abnormal keratinization occurring in human skin after prolonged edema may have a similar cause to the keratin changes produced by experimental saline injections in mouse tails.

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